Actinomadura catellatispora sp. nov. and Actinomadura glauciflava sp. nov., from a sewage ditch and soil in southern China

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Two soil isolates, strains 80-60T and 3.24T, were shown to have chemical and morphological properties consistent with their classification in the genus Actinomadura. The almost complete 16S rDNA sequences generated for the two organisms were aligned with available sequences of representatives of the genus Actinomadura and related taxa. It was apparent from the resultant phylogenetic trees that each of the strains formed a distinct phyletic line within the evolutionary radiation occupied by the genus Actinomadura. The two organisms could also be distinguished from one another and from representatives of all the validly described species of Actinomadura using a set of phenotypic properties. It is proposed that strains 3.24T (= AS 4.1522T = IFO 16341T) and 80-60T (= AS 4.1202T = IFO 14668T = JCM 16161T) be classified in the genus Actinomadura as Actinomadura catellatispora sp. nov. and Actinomadura glauciflava sp. nov., respectively.

INTRODUCTION

Phylogenetic analyses using sequences of 16S rDNA, 23S rRNA and 16S–23S rRNA gene spacers have helped to clarify relationships between members of the family Thermomonosporaceae (Stackebrandt et al., 1997; Zhang et al., 1998, 2001). This taxon currently includes the genera Actinocorallia Linuma et al. 1994, Actinomadura Lechevalier and Lechevalier 1968, Spirillospora Couch 1963 and Thermomonospora Henssen 1957, the type genus. Members of these taxa share a range of chemotaxonomic and molecular systematic properties that distinguish them from other actinomycetes (Zhang et al., 1998, 2001; Miyadoh & Miyara, 2001), including near relatives classified in the families Nocardiopsaceae (Rainey et al. 1996) emend. Zhang et al. 2001 and Streptosporangiaceae (Goodfellow et al. 1990) emend. Stackebrandt et al. 1997.

Organisms previously classified as Nocardia dassonvillei, Nocardia madurae and Nocardia pelletieri were the founder members of the genus Actinomadura (Lechevalier & Lechevalier, 1968), though Actinomadura dassonvillei was subsequently reclassified as Nocardiopsis dassonvillei (Meyer, 1976). Following its introduction, the genus Actinomadura became a dumping ground for an assortment of aerobic, Gram-positive, non-acid-fast, non-motile sporocinomycetes that had cell walls rich in meso-diaminopimelic acid (meso-A2pm), but lacking diagnostic sugars (wall chemo-type III sensu Lechevalier & Lechevalier, 1970). The application of chemotaxonomic, molecular systematic and numerical phenetic procedures clearly showed that the genus was in need of taxonomic revision (Athalye et al., 1985; Fischer et al., 1983; Poscher et al., 1985; Kroppenstedt et al., 1990), a task that was initiated by the extensive studies of Zhang et al. (1998, 2001).

The revised genus Actinomadura accommodates aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that typically form non-fragmenting, extensively branched substrate mycelia and aerial hyphae that differentiate into short to long, straight, hooked or spiral chains of spores with either folded, irregular, smooth, spiny or warty spores. Members of the genus Actinomadura contain meso-A2pm, the sugars galactose, glucose, madurose, mannose and ribose, major proportions of hexahydrogenated menaquinones with nine isoprene units, complex fatty acids, including hexadecanoic, 14-methylpentadecanoic and
10-methylotadecanonic acids as predominant components and diphaspatidylglycerol and phosphatidylinositol as major phospholipids (Kroppenstedt et al., 1990).

The genus presently contains 28 validly described species, though there is evidence that Actinomadura spadix may merit generic status (Athalye et al., 1985; Ochi et al., 1991; Zhang et al., 2001). Further comparative studies are also needed to establish clear relationships between Actinomadura echinospora, Actinomadura umbrina and Thermomonospora curvata (the type species of the genus), as these organisms are well separated from other Actinomadura species on the basis of 16S and 23S rDNA sequence data (Zhang et al., 2001). Phylogenetic data also indicate that Spirillospora albida, the type species of the genus, has a very close evolutionary relationship with some Actinomadura species.

The aim of the present study was to determine the taxonomic status of two soil isolates, strains 3.24T and 80-60T, which were considered to have phenotypic properties typical of actinomadurae. Isolate 80-60T was invalidly described as ‘Streptomyces glaucoflavus’ by Zhang et al. (1984), but was subsequently shown to have chemical and morphological properties typical of Actinomadura strains (Itoh et al., 1987). The two organisms were examined for a range of genotypic and phenotypic properties and were found to form new centres of taxonomic variation within the genus Actinomadura; the names Actinomadura catellatispora sp. nov. and Actinomadura glauciflava sp. nov. are respectively proposed for strains 3.24T and 80-60T.

**METHODS**

**Isolation, maintenance and cultivation.** Strain 3.24T was isolated on plates of glucose-asparagine agar (glucose, 10 g; l-asparagine, 0.5 g; K2HPO4, 0.5 g; agar, 15 g; distilled water, 1 l; pH 7.2-7.4) seeded with a soil suspension and incubated at 28 °C for 7 days. The soil suspension was prepared from a mud sample taken from a sewage ditch in Zhanjiang City, Canton Province, southern China. Similarly, strain 80-60T was isolated, under the same conditions, using a soil suspension prepared from a soil sample collected at the Institute of Tropical Plants, Xishuang Bana, Yunnan Province, southern China, as described by Zhang et al. (1984). The isolates were maintained as glycerol suspensions (20 %, v/v), as were the type strains of Actinomadura citrea, Actinomadura coerules, Actinomadura latina, Actinomadura livida, Actinomadura luteofluorescens, Actinomadura nitritigenes and Actinomadura verrucosospora. Biomass for chemotaxonomic and molecular systematic studies was prepared by growing all of the organisms in shake flasks of modified Sauton’s broth (Mordarska et al., 1972) at 28 °C for 7 days. Cells for chemotaxonomic analyses (isolates 3.24T and 80-60T) were washed twice in distilled water and freeze-dried; those for molecular systematic studies (all strains) were washed in NaCl/EDTA buffer (0-1 M EDTA, pH 8.0: 0-1 M NaCl) and stored at −20 °C until required.

**Cultural and morphological studies.** The undisturbed arrangement of the aerial hyphae and spore chain morphology of the isolates was observed from cultures grown on glucose-yeast extract-malt extract agar (ISP 2 medium; Shirling & Gottlieb, 1966) at 28 °C for up to 4 weeks, using the cover-slip technique of Kawai & Shinobu (1959). Growth on the cover-slip was fixed and examined following the procedure described by Zhou et al. (1998). Spore ornamentation was observed by examining gold-coated, dehydrated preparations, using a Hitachi 5-570 SEM.

**Biochemical and physiological properties and chemotaxonomy.** The isolates were examined for a broad range of phenotypic properties as described by Athalye et al. (1985).

The diagnostic isomers of A₂pm, the predominant menaquinones, sugars, polar lipids and the DNA base composition of the isolates were determined as described by Lu et al. (2001).

**DNA extraction and 16S rDNA sequencing.** Standard procedures were used to extract genomic DNA from all of the test organisms (Pitcher et al., 1989; Kim et al., 1998).

PCR amplification of 16S rDNA samples prepared from the isolates and from the type strains of A. latina and A. nitritigenes was carried out as described previously (Kim et al., 1996). The PCR products were purified according to the Wizard PCR purification system (Promega) and then sequenced using a DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and universal primers as described previously (Lu et al., 2001). Sequence gel electrophoresis was carried out and nucleotide sequences were obtained automatically using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

**Analysis of sequence data.** The almost complete 16S rDNA sequences of the organisms were aligned manually with corresponding sequences of representatives of the family Thermomonosporaceae retrieved from the DDBJ/EMBL/GenBank databases using the program PHYDIT (J. Chun, unpublished data). Evolutionary trees were inferred using the least-squares, maximum-likelihood, maximum-parsimony and neighbour-joining treeing algorithms from the PHYLIP suite of programs (Felsenstein, 1993) and evolutionary distance matrices were generated for the least-squares and neighbour-joining methods as described by Jukes & Cantor (1969). The stability of the groupings was evaluated by bootstrap analyses (1000 replicates) of the neighbour-joining dataset using the programs SEQBOOT and CONSENSE (Felsenstein, 1993).

**DNA–DNA relatedness studies.** Levels of DNA–DNA relatedness between isolate 80-60T and the type strains of A. citrea, A. coerules, A. luteofluorescens, Actinomadura macra and A. verrucosospora were determined, in duplicate, following the procedure described by De Ley et al. (1970).

**RESULTS AND DISCUSSION**

Almost complete 16S rDNA sequences were generated for isolates 3.24T and 80-60T and for the type strains of A. latina and A. nitritigenes (>1445 nt). Comparison of these sequences with those of representatives of the family Thermomonosporaceae sensu Zhang et al. 2001 showed that all of the strains fall within the range of the evolutionary radiation occupied by the genus Actinomadura (data not shown). The isolates also exhibited chemical markers typical of members of this taxon, i.e. they contained: meso-A₂pm as the wall diamino acid; the sugars galactose, glucose, madurose, mannose and ribose; hexahydrogenated menaquinones with nine isoprene units as the predominant isoprenologe (~80 %), minor amounts (~10–12 %) of tetra- and octahydrogenated components with nine isoprene units, diphaspatidylglycerol and phosphatidylinositol as major polar lipids; and complex mixtures of fatty acids,
notably major proportions of hexadecanoic (~40% of total fatty acids), 14-methylpentadecanoic (~20%) and 10-methyloctadecanoic (~20%) acids. These chemical properties distinguish the isolates from members of the family Thermomonosporaceae, apart from those classified in the genus Actinomadura (Kroppenstedt et al., 1990; Miyadoh & Miyara, 2001). In addition, strains 3.24^T and 80-60^T have G+C-rich DNA (70.8 and 72.0 mol%, respectively), but lack mycolic acids.

The phenotypic properties of the isolates are also consistent with their classification in the genus Actinomadura. The organisms are aerobic, non-motile, Gram-positive, non-acid/alcohol-fast actinomycetes that form extensively branched, non-fragmenting substrate mycelia. Isolate 80-60^T produces abundant bluish-green aerial hyphae on ISP 2 medium (Shirling & Gottlieb, 1966) that differentiate into short, curved or hooked or spiral (one turn) spore chains with a warty ornamentation (Fig. 1a). In contrast, strain 3.24^T formed short, straight chains of smooth-surfaced spores on the aerial mycelium (Fig. 1b).

The positions of the tested strains in the 16S rDNA Actinomadura tree are shown in Fig. 2. Strain 3.24^T is most closely related to the type strain of A. livida; the two organisms share 96.8% 16S rDNA similarity, which corresponds to 45 nt differences at 1450 locations. Much higher 16S rDNA similarities have been found between representatives of validly described species of Actinomadura. The type strains of A. citrea and A. coerulea, for instance, share 99.4% similarity, which corresponds to 8 nt differences at 1450 sites. Representatives of these taxa have a DNA–DNA relatedness value of 48% (Poscher et al., 1985), a value well below the 70% cut-off point recommended by Wayne et al. (1987) for the delineation of genomic species. It is also apparent from Fig. 2 that strain 80-60^T belongs to the A. luteofluorescens subclade. The taxonomic integrity of this group is supported by a bootstrap value of 61% and by

Fig. 1. SEM of strain 80-60^T (a), showing hooked and spiral chains of warty ornamented spores, and strain 3.24^T (b), showing short chains of smooth ornamented spores, after 4 weeks growth at 28°C on ISP 2 medium. Bars, 1 μm.

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences of strains 3.24^T and 80-60^T and representatives of validly described species of Actinomadura. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Kluge & Farris, 1969) treeing algorithms; the symbols F and P respectively indicate branches recovered using the least-squares and maximum-parsimony methods. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar, 0.02 substitutions per nucleotide position. A., Actinomadura; S., Spirillospora; T., Thermomonospora.
results obtained with all four treeing algorithms. Strain 80-60\textsuperscript{T} is most closely related to the type strain of \textit{A. citrea}. The two organisms share 98.4\% 16S rDNA similarity, which corresponds to 23 nt differences at 1450 sites. DNA–DNA relatedness studies are now commonly used to resolve the finer taxonomic relationships between representatives of such closely related species. In the present investigation, it is evident that isolate 80-60\textsuperscript{T} belongs to a distinct genomic species, as it shares mean DNA–DNA relatedness values of 62\% with \textit{A. luteofluorescens} JCM 4491\textsuperscript{T}, 53\% with \textit{A. citrea} JCM 3295\textsuperscript{T} and \textit{A. verrucospora} JCM 3147\textsuperscript{T} and 38\% with \textit{A. coerulea} JCM 3320\textsuperscript{T}. These strains can also be distinguished from one another, and from isolate 3.24\textsuperscript{T}, using a range of phenotypic properties (Table 1).

It is evident from the genotypic and phenotypic data that isolates 3.24\textsuperscript{T} and 80-60\textsuperscript{T} form the core of two novel species within the genus \textit{Actinomadura}. It is therefore proposed that these organisms be given the names \textit{Actinomadura catellatispora} sp. nov. and \textit{Actinomadura glauciflava} sp. nov., respectively. It is also clear from the phylogenetic data that \textit{A. latina} DSM 43382\textsuperscript{T} and \textit{A. nitritigenes} DSM 44132\textsuperscript{T} form distinct phyletic lines, thereby underpinning the taxonomic status of these species, which were circumscribed mainly on the basis of phenotypic data (Trujillo & Goodfellow, 1997; Lipski & Altendorf, 1995).

**Description of \textit{Actinomadura catellatispora} sp. nov.**

\textit{Actinomadura catellatispora} (ca.tel.la.ti.spo’ra. L. n. \textit{catella} small chain; Gr. n. spor\textit{a} a seed; N.L. fem. n. \textit{catellatispora} organism forming small chains of spores).

Aerobic, Gram-positive, non-acid/alcohol-fast, non-motile actinomycete that forms an extensively branched, non-fragmenting, light-yellow substrate mycelium on glucose-yeast extract-malt extract agar (ISP 2 medium). Short chains of spores (0-85 \textmu m diameter) are formed on the aerial mycelium. The spore surface is smooth and the aerial spore mass is yellow. Diffusible pigments are not formed. Aesculin and gelatin are degraded but not casein, hypoxanthine, starch, tyrosine or xanthine. Nitrate is reduced. The organism is chemo-organotrophic, has an oxidative type of metabolism and grows at temperatures between 18 and 35 °C. Isolated from a mud sample taken from a sewage ditch in Zhanjiang City, Canton Province, southern China. The type strain is isolate 3.24\textsuperscript{T} (= AS 4.1522\textsuperscript{T} = IFO 16341\textsuperscript{T}), which has a DNA G+C content of 70-8 mol\%. The species description is based upon a single strain and hence serves as the description of the type strain.

**Description of \textit{Actinomadura glauciflava} sp. nov.**

\textit{Actinomadura glauciflava} (glau’ci fla’va. L. fem. adj. \textit{glauc}a bluish-green; L. adj. \textit{flaveus} yellow; N.L. adj. \textit{glauciflava} bluish-green yellow).

The description is based on data taken from this and earlier studies (Zhang \textit{et al}, 1984; Itoh \textit{et al}, 1987). Aerobic, Gram-positive, non-acid/alcohol-fast, non-motile actinomycete that forms an extensively branched, non-fragmenting, substrate mycelium that carries aerial hyphae which differentiate into curved or hooked or spiral chains of spores (1 \textmu m diameter) on glucose-yeast extract-malt extract agar (ISP 2 medium). The spore surface is warty,

### Table 1. Phenotypic characters that distinguish strains 3.24\textsuperscript{T} and 80-60\textsuperscript{T} from the type strains of closely related \textit{Actinomadura} species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore chain arrangement</td>
<td>Straight</td>
<td>Hooks, spirals</td>
<td>Hooks</td>
<td>Hooks</td>
<td>Hooks, spirals</td>
<td>Hooks</td>
<td>Hooks, spirals</td>
</tr>
<tr>
<td>Spore surface ornamentation</td>
<td>Smooth</td>
<td>Warty</td>
<td>Irregular</td>
<td>Warty</td>
<td>Irregular</td>
<td>Warty</td>
<td>Warty</td>
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<tr>
<td>Growth on ISP 2 medium:</td>
<td>Yellow</td>
<td>Blush-green</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>White-yellow</td>
<td>White</td>
</tr>
<tr>
<td>Aerial mycelium</td>
<td>Light yellow</td>
<td>Yellow-brown</td>
<td>Yellow-brown</td>
<td>Brown</td>
<td>Brown</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Substrate mycelium</td>
<td>None</td>
<td>Yellow</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Yellow</td>
<td>None</td>
</tr>
<tr>
<td>Diffusible pigment</td>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Casein</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hypoxanthine</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Starch</td>
<td>–</td>
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<td>–</td>
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<td>Tyrosine</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Xanthine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
</tbody>
</table>
the aerial spore mass is bluish-green. A yellow diffusible pigment is produced. Aesculin, casein, gelatin, starch and hypoxanthine are degraded, but not tyrosine or xanthine. Nitrate is reduced. The organism is chemo-organotrophic, has an oxidative type of metabolism and grows at temperatures between 18 and 35 °C. Isolated from soil collected in Yunnan Province, southern China. The type strain is isolate 80-60T (= AS 4.1202T = IFO 14668T = JCM 16161T), which has a DNA G+C content of 72 mol%. The species description is based on a single strain and hence serves as the description of the type strain.

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